

**CREATING A LIBRARY OF GENETICALLY ENCODED HEME SENSORS
WITH VARYING BINDING AFFINITIES**

A Thesis
Presented to
The Academic Faculty

By

Jessie David Ashworth

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**CREATING A LIBRARY OF GENETICALLY ENCODED HEME SENSORS
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Approved by:

Dr. Amit R. Reddi, Advisor
School of Chemistry
Georgia Institute of Technology

Dr. Christoph Fahrni
School of Chemistry
Georgia Institute of Technology

Dr. Raquel Lieberman
School of Chemistry
Georgia Institute of Technology

Date Approved: December 8, 2016

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LIST OF ABBREVIATIONS

Abbreviation or Symbol	Term
AD	Alzheimer's disease
ALA	δ -Aminolevulinic acid
Ala	Alanine
Asn	Asparagine
A β	Amyloid- β protein
CFP	Cyan fluorescent protein
Cu	Copper
Cys	Cysteine
Cyt <i>b</i> ₅₆₂	Cytochrome <i>b</i> ₅₆₂
<i>E. coli</i>	<i>Escherichia coli</i>
EGFP	Enhanced green fluorescent protein
EV	Empty vector yeast cells
FRET	Förster resonance energy transfer
<i>hem1</i> Δ	Yeast without <i>hem1</i> encoding ALA synthase
His	Histidine
HS1	Heme sensor 1
HS1-M7A	Heme sensor 1 with Met7Ala mutation
LH	Labile heme pool
Lys	Lysine
Met	Methionine

mKate2	Far-red fluorescent protein
NO	Nitric oxide
OD ₆₀₀	Optical density at 600 nm
PBS	Phosphate-buffered saline
R	Fluorescence intensity ratio
ROS	Reactive oxygen species
SA	Succinylacetone
Ser	Serine
Tyr	Tyrosine
WT	Wild-type yeast cells
Yeast	<i>Saccharomyces cerevisiae</i>
YFP	Yellow fluorescent protein
Zn	Zinc

SUMMARY

Heme is an important biological metallo-cofactor that also works as a signaling molecule in cells. Despite its importance, heme trafficking and mobilization in the cell are currently not well understood, in part due to the limited ability to visualize and quantify heme *in vivo*. This inconvenience has recently been overcome by the development of genetically encoded heme sensors by the Reddi lab. Using these sensors, the Reddi lab has identified new heme trafficking factors and probed the spatio-temporal dynamics of heme mobilization¹. While the heme sensors are powerful tools for imaging and quantifying heme, improvements could be implemented that would allow for greater utility than is currently available due to particular limitations in the prototype sensors. A current limitation in heme sensing is that the heme dissociation constants of the prototype sensors span a limited range. The high affinity heme sensor, HS1 ($K_d^{\text{II}} \sim 10$ pM and $K_d^{\text{III}} = 9$ nM) is quantitatively saturated in all sub-cellular compartments, including the cytosol, nucleus, and mitochondria, making it unsuitable for heme monitoring. The low affinity sensor, HS1-M7A ($K_d^{\text{II}} = 20$ nM and $K_d^{\text{III}} = 2$ uM) is ~20-50% saturated with heme in the cytosol, but ~0 % saturated in the nucleus and mitochondria, making it unsuitable for heme monitoring in these compartments. In an effort to broaden the utility of the heme sensors, we report herein an expanded library of sensors engineered to span a wide-array of heme affinities. Heme binding residues were mutated to Ala, Cys, His, Lys, Met, Ser and Tyr to generate a panel of 47 new mutants which were found to be between 0 and 100% saturated when expressed in the yeast cytosol. These new tools will enable

unprecedented access to cellular heme pools for probing heme homeostasis in health and disease.

CHAPTER 1

INTRODUCTION

1.1 Heme

Heme (iron protoporphyrin IX) is an important metallo-cofactor of numerous proteins and consists of an iron center enclosed in an organic tetrapyrrole ring. It is used in a wide range of proteins with varying functions including electron transfer,² gas transport,³ and gas sensing.⁴ Heme, while being vital to life, is also toxic to cells because of its ability to produce reactive oxygen species (ROS) which cause lipid peroxidation.⁵ This is not an issue when considering the largest heme pool, which is the exchange-inert heme that is bound to heme-proteins, because this heme is tightly bound and unavailable to produce oxygen radicals. In recent years however it has been shown that a labile heme pool is present in the cell for signaling and other processes.⁶ This dichotomy of heme requires that it must be tightly regulated and buffered in the cell to prevent oxidative damage, while still being available for signaling and mobilization throughout the cell. These facts bring about a fundamental question: how is heme regulated in the cell? This question quickly becomes complex due to many unanswered questions, such as, what is buffering heme and how much labile heme resides in different compartments of the cell, or what is transporting heme within the cell? In an effort to help answer these questions the work described in this thesis builds upon a previously developed heme sensor to create sensors with an expanded range of binding affinities.

1.2 Regulation and Transport of Heme

Heme is synthesized through the Shemin pathway, seen in Figure 1, starting in the mitochondrial matrix with the production of 5-aminolevulinate (ALA), through the condensation of glycine and succinyl-CoA.⁷ ALA is then transported outside the mitochondrial matrix where it goes through three condensation reactions to produce Uroporphyrinogen III.⁷ Uroporphyrinogen III is then partially decarboxylated to form Coproporphyrinogen III.⁷ Coproporphyrinogen III is then transported to the intermembrane space where it goes through an oxidative decarboxylation forming two vinyl groups on the molecule, and one further oxidation that yields protoporphyrin IX back inside the mitochondrial matrix.⁷ The final step is the insertion of Fe^{2+} into the protoporphyrin ring.⁷ At this point a fully developed heme molecule is available to be shuttled throughout the cell. This is where the current understanding of heme dynamics comes into play.

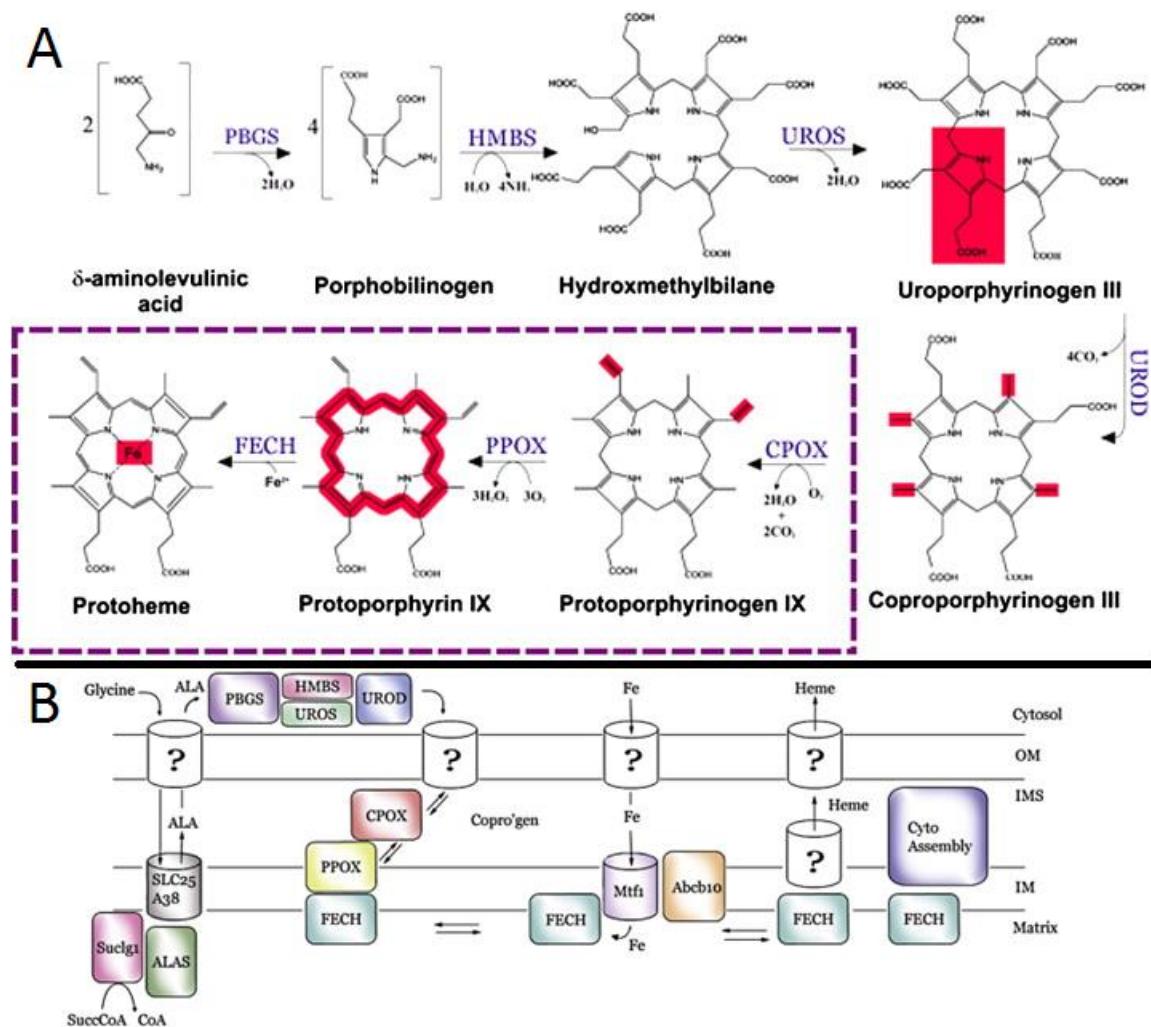


Figure 1. A - Heme synthesis pathway beginning with 5-aminolevulinat. **B** – Proposed machinery of heme synthesis with experimentally undefined components labeled with a “?”. Reprinted (adapted) with permission from Hamza et al. Copyright Elsevier 2012.⁷

It is currently known that heme is required in virtually every organelle in the cell; however, the process by which heme traverses the cell from the mitochondria to the organelles remains elusive.⁸ The toxic effects of heme requires it to be buffered in some way to prevent from oxidative damage and cell death that would incur while moving throughout the cell, which leads to the question of how is heme transported throughout the cell? Currently there are two prevailing ideas when it comes to how heme moves

through the cell. The first is that heme is transported in specialized vesicles around the cell creating a sub environment capable of limiting the oxidative stress of the heme.⁹ The second is that specialized proteins called heme chaperones are capable of buffering and carrying heme from place to place in the cell, while preventing heme's production of ROS.⁷ It stands to reason that perhaps both of these mechanisms are in play to mitigate the possibility of oxidative stress. The assumed pathway by which heme is shuttled throughout the cell can be seen in Figure 2, in which one will notice that most of the suspected transporters and chaperones of heme are currently unknown. Development of heme sensors capable of being targeted to organelles and sensing changes in labile heme (LH) will be vital in further understanding of how heme is transported in the cell and uncovering the proteins involved in these processes. This statement is bolstered by the fact that a possible heme buffering protein was discovered using the first generation heme sensor that is subject for further development in this thesis. While the venture to uncover heme regulation and transportation is noble on its own, it is also important in understanding the role of heme in disease.

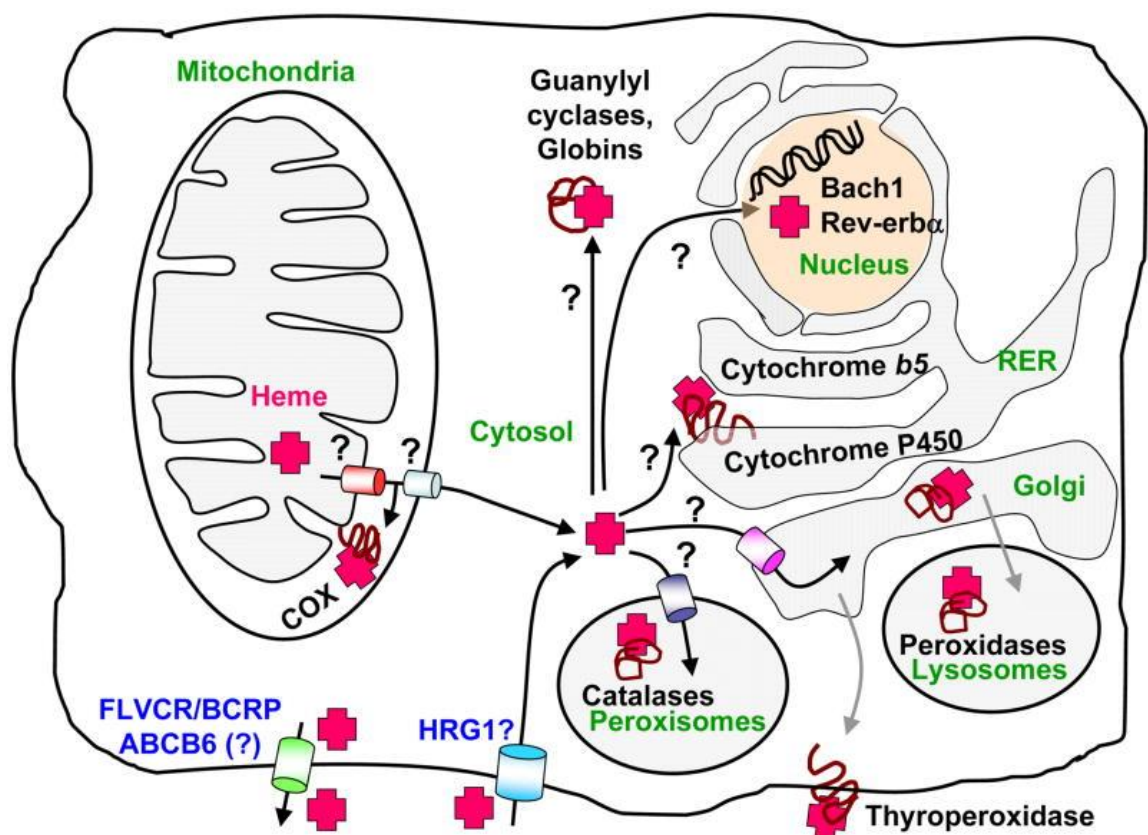


Figure 2. Proposed model of heme transport in the cell. Heme (Red Cross) is shown traversing the cell to arrive at the different organelles in which it is needed, while pathways taken to reach each organelle is depicted with an arrow. Unknown pathways are indicated with a Black arrow and a “?” while known pathways are grey. Reprinted (adapted) with permission from Severance et al. Copyright 2009 American Chemical Society.⁸

1.3 Heme and Diseases

Heme has been linked to many diseases, including neurodegenerative diseases,¹⁰ colon cancer,¹¹ and vascular diseases.¹² The absolute role of heme in disease is still being investigated; however, its ability to produce ROS and integrate with cell membranes is at the core of what makes heme a suspect in many diseases. This can be seen with the suspected role that heme plays in both Alzheimer’s disease as well as in colon cancer.

Alzheimer's disease (AD) is the most common neurodegenerative disorder in the United States, affecting ~ 5 million people in 2013, and is the 5th leading cause of death in people over the age of 65.¹³ AD is distinguished by the buildup of senile plaque, which is composed of amyloid- β (A β) and Tau proteins, in the brain.¹⁴ Heme metabolism and regulation is altered in AD brains¹⁴ alongside proteins that interact with heme, such as heme oxygenase.¹⁵ Because of this, heme has been suspected to play several roles in the development of AD. It has been shown that heme can interact with A β , alongside Cu, to produce a peroxidase complex capable of generating ROS that could lead to the oxidative stress and death of neuronal cells in AD.¹⁶ Heme has also been shown to prevent the aggregation of A β plaques, allowing for A β monomers to more easily traverse brain tissue.¹⁷ The effects of this are not well understood, but it could be that heme is compounding the problem of AD in the brain by allowing smaller A β monomers to spread through the brain better than the aggregates found in plaque, while also producing ROS that are causing cytotoxicity and cell death.

Colon cancer is one of the leading causes of cancer in the United States, and other Western countries.¹⁸ While some genetic factors have been shown to be important in the development of colon cancer, it is clear that environmental factors are key to the development of the disease.¹⁸ Heme has long been suspected as a key contributor to the development of colon cancer. This is supported by numerous epidemiological studies, which underscored the high meat content in Western diets compared to the limited meat diets of Asian countries. More recent rodent studies however have shown a more direct link to heme in the development of colon cancer. The mechanism by which heme may induce colon cancer is still unclear; however, there are several ideas as to how it may be

occurring. The first explanation for heme's role in the progression of colon cancer is that heme increases the redox stress on the colon which in turn causes DNA damage and increased hyperproliferation.¹¹ More recent studies are suggesting that this explanation may be somewhat too simplistic. It has recently been suggested that heme is altering the microbiota of the gut that in turn is increasing progression of colon cancer.¹⁹ This result shows that heme may play a more metabolic role in the development of colon cancer while also providing the oxidative environment capable of causing the DNA damage that ultimately leads to colon cancer.

In all of these cases, our understanding of how heme affects the progression of the disease is limited by the limited knowledge of heme regulation and the inability to visualize and quantify labile heme in live cells.

1.4 Sensing Heme

Sensors for the detection of metal ions in biology have flourished in recent years with dozens of sensors being developed for a wide range of metals.²⁰ Development of both small molecule and genetically encoded protein-based sensors has allowed researchers to study transition metal ions, particularly zinc and copper, with high sensitivity and spatial resolution that were previously impossible at the single cell level.²⁰

Imaging and quantifying heme in cells using fluorescent sensors represents a challenging task. Compared to fluorescent probes for transition metal ions, the creation of genetically encoded sensors, capable of high contrast imaging of heme in the cell, is particularly challenging. This is in part due to the ability of heme to act as a fluorescence quencher through Förster resonance energy transfer (FRET). This significantly limits the

ability to create heme sensors using cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) FRET pair, which have been commonly employed for the design of genetically encoded metal ion sensors.²⁰ This problem necessitates that any sensor used to detect heme by a FRET approach must use a fluorescent protein outside the range of heme absorbance and/or have heme binding occur significantly far from the fluorescent proteins to increase sensitivity of the sensor and intensity of the signal. In light of this, two separate sensors have been developed in an effort to create a cellular heme sensor.

Song et al. developed a sensor that uses FRET between CFP and YFP to detect heme.²¹ These fluorescent proteins are linked with two heme transfer proteins between them, which allow for CFP and YFP to be brought together in the presence of heme and allow for FRET to occur.²¹ Because of its seemingly modular setup and ease of use in investigation, this technique has been previously used for the design of numerous metal ion sensors.²⁰ Significant differences in LH levels were detected in a range of different mammalian cancer cell lines and in different compartments of the cell.²¹

A somewhat different approach was taken by the Reddi lab at Georgia Tech, who developed a genetically encoded heme sensor that uses FRET between enhanced green fluorescent protein (EGFP) and heme as a signal for sensing LH in the cell.¹ In doing this the sensor uses the ability of heme to quench fluorescence as part of its readout while providing a heme-independent readout with mKATE2 to provide a ratiometric sensor.¹

The basic framework of the sensor is an EGFP- cytochrome *b₅₆₂* (cyt *b₅₆₂*) fusion protein developed by Arpino et al.²² This fusion protein was created using combinatorial mutagenesis which ultimately yielded CG6, a mutant EGFP with Cyt *b₅₆₂* inserted

between residue 39 and 40.²² This scaffold places the fluorophore of EGFP within ~ 17 Å of heme which is enough to give ~100% energy transfer when heme is bound to Cyt *b*₅₆₂.²² This energy transfer gives an Off/On signal that indicates heme bound or unbound. Purified CG6 can be used to study, for example, the *in vitro* heme transfer between proteins or to determine the binding affinity of heme-binding proteins based on competition titrations. However, application of the probe for *in vivo* experiments is limited due to the intensity based readout obtained from this sensor rather than a ratiometric readout. A ratiometric readout is preferred when experimenting *in vivo* because effects of sensor concentration, bleaching and several other factors can more easily be corrected.

This leads to the next evolution of CG6, which is the addition of mKate2 to produce a ratiometric readout that can be used in the cell for quantifying buffered heme levels. mKate2 is a red fluorescent protein that has an emission spectrum at 620 nm, just outside the range of heme absorbance. This alongside the fact that the mKate2 Förster distance is approximately double that of eGFP's suggests that mKate2 fluorescence is independent of heme. This new sensor was given the name HS1 and was the first iteration of a genetically encoded ratiometric sensor for the detection of heme. The structure of this sensor can be seen in Figure 3. In order to obtain useful data one must take the ratio of eGFP fluorescence and dividing by the mKate2 fluorescence giving the eGFP/mKate2 ratio. As heme binds to HS1 eGFP fluorescence decreases, lowering the overall eGFP/mKate2 ratio, therefore larger ratio values indicate low concentrations of heme while smaller ratio values indicate high heme concentrations.

It was determined, through titration and *in situ* studies of HS1, that HS1 was binding heme too tightly ($K_d^{\text{II}} < 1\text{nM}$)¹ to be useful in cellular studies and so a mutation was made in hopes of creating a sensor with a weaker binding affinity to heme. This mutation was a HS1-M7A and was altered by replacing the Met₇ axial binding ligand of Cyt *b*₅₆₂ with an Ala. The affinity of HS1-M7A ($K_d^{\text{II}} \sim 25\text{nM}$)¹ appears to be in the range capable of detecting heme in the cytosol of yeast. These sensors were validated with several *in vitro* tests that were completed to make sure that the sensors were only detecting changes in heme, that the sensors were not pH dependent, and that heme binding was reversible.¹ Once the sensors were determined to be valid they were used to probe heme regulation in the cytosol as well as being targeted to the nucleus and the mitochondria.

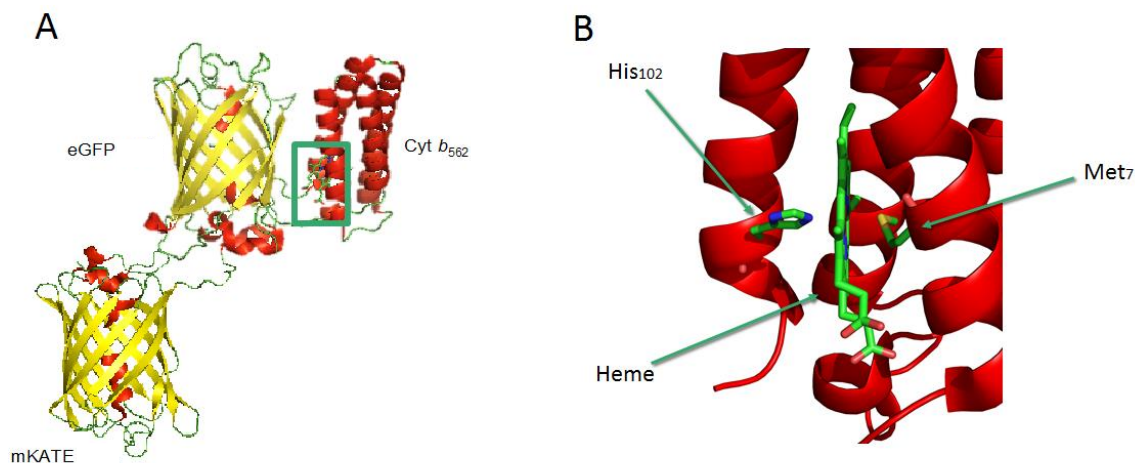


Figure 3. **A** – Ribbon structure of HS1 with each domain labeled. The green box indicates the heme binding site of HS1. **B** – Heme binding site of HS1 with heme and the two axial coordinating sites (His₁₀₂ and Met₇) indicated. Models are derived from the X-ray structures of mKATE (PDB: 3BXB) and CG6 (PDB: 3U8P).^{23,22}

Using these sensors, the cytosolic LH pool was determined to be ~20-40 nM in yeast¹ which is in line with the previously proposed 10-100 nM range.²⁴ From Figure 4, it can also be seen that the LH pool exists in three different states in the cytosol of yeast.¹ This was a novel observation suggesting that the LH pool may exist in a state of flux rather than remaining at a rather constant concentration, lending to the idea of heme being used as a signaling molecule.

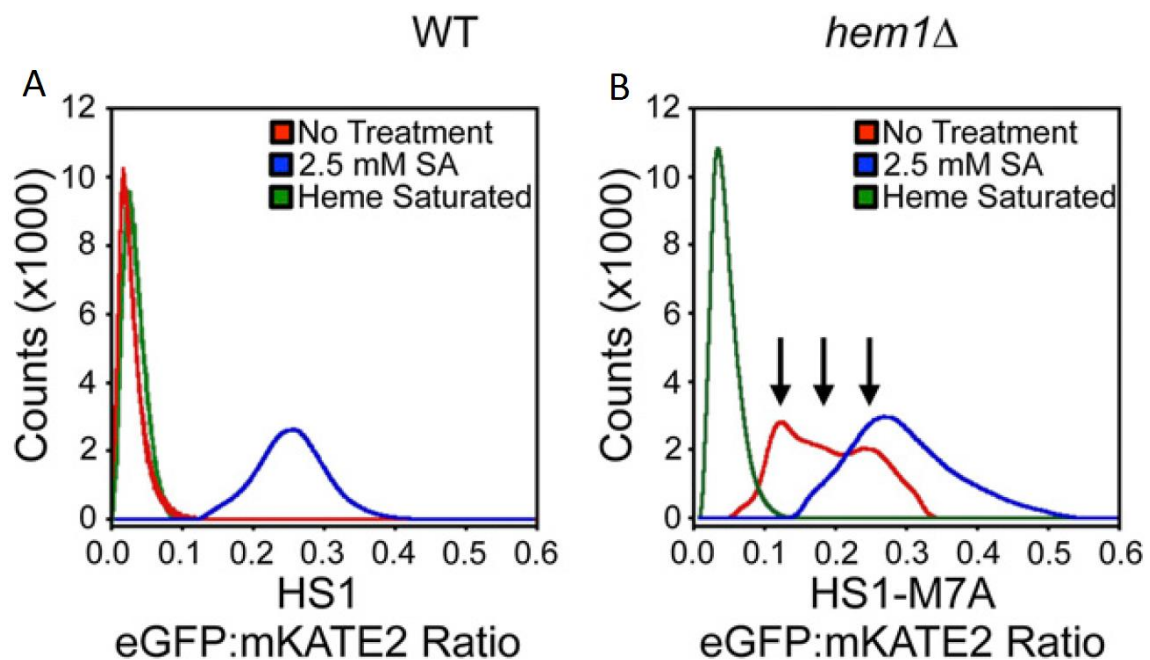


Figure 4. Analysis of cytosolic heme using flow cytometry with HS1 and HS1-M7A. Green lines indicate saturated sensor. Red lines indicate normal yeast growth conditions. Blue lines indicate growth with succinylacetone (SA), which prevents heme production. Reprinted (adapted) with permission from Hanna *et al.* Copyright 2016 National Academy of Sciences.¹

As can be seen in Figure 5, nitric oxide (NO) was also shown to have a significant effect on cytosolic and nuclear heme pools, which is in line with previous studies.²⁵

Glyceraldehyde phosphate dehydrogenase (GAPDH) was shown to have heme buffering capabilities as well as the ability to regulate the heme-dependent transcription factor Hap1p.¹

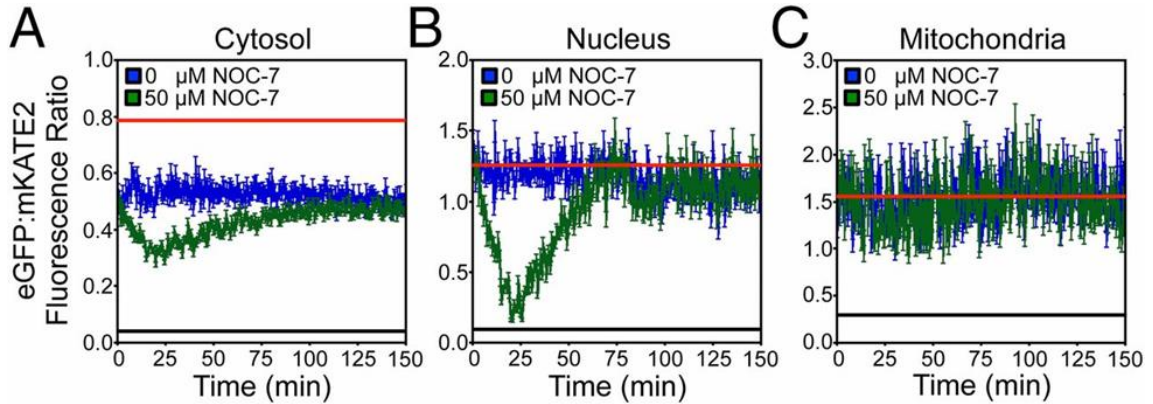


Figure 5. Fluorescence ratio of HS1-M7A over time with (Green) and without (Blue) the addition of NOC-7 (NOC-7 releases NO molecules when added). Red lines indicate fluorescence ratio when HS1-M7A is 0% saturated while black lines are 100% saturated. Reprinted (adapted) with permission from Hanna *et al.* Copyright 2016 National Academy of Sciences.¹

While the utility of HS1 and HS1-M7A cannot be denied when looking at the previous results, the limitations of the current generation of sensors can already begin to be seen. The restricted binding affinity of these sensors limits accurate detection of heme in various compartments of the cell, such as the nucleus and the mitochondria. This problem may be compounded when trying to insert sensors into new cell lines that span a wide range of labile heme concentrations. With only two binding affinities available it is clear that creating a library of sensors with varying heme binding affinities will be required for future experimentation. Also the inability to detect oxidized heme may limit

the capacity for these sensors to fully uncover heme dynamics in the cell. As such, the development of a sensor with oxidation state specificity may improve our understanding of heme mobilization in cells.

CHAPTER 2

SENSOR DESIGN AND SCREENING SENSORS

2.1 Introduction

The prototype heme sensors, HS1 and HS1-M7A, are powerful tools for the monitoring and quantification of exchange labile heme.¹ As described in section 1.4, these sensors have provided unprecedented access to heme pools relevant for trafficking and signaling. However, the affinities of these sensors are such that they are not adequate for heme sensing in subcellular compartments like the nucleus and mitochondria. HS1 ($K_d^{\text{II}} \sim 10$ pM and $K_d^{\text{III}} = 9$ nM) is quantitatively saturated in all sub-cellular compartments, including the cytosol, nucleus, and mitochondria, making it unsuitable for heme monitoring. The low affinity sensor, HS1-M7A ($K_d^{\text{II}} = 20$ nM and $K_d^{\text{III}} = 2$ uM) is ~20-50% saturated with heme in the cytosol, but has no heme bound to it in the nucleus and mitochondria, making it unsuitable for monitoring labile heme concentration and dynamics in these compartments. In order to expand the range of heme sensing, we generated a library of ~47 heme binding site mutations within the prototype HS1 sensor and screened for changes in heme saturation within the yeast cytosol. This approach resulted in 6 new HS1 variants that span a range of heme binding affinities between that of HS1 and HS1-M7A, generating new candidate sensors that may be ideal for heme imaging in subcellular compartments like the nucleus and mitochondria.

The changes in free energy associated with heme binding to the heme binding domain, Cyt *b*₅₆₂, arise from a combination of factors, including iron coordination by axial ligands, protein-porphyrin hydrophobic interactions, and electrostatic interactions

between the heme propionates and charged residues of the protein. Our initial strategy to expand the range of sensor heme binding affinities relied on altering the heme-iron axial ligands. Cyt *b*₅₆₂ employs Met₇ and His₁₀₂, to coordinate heme.²⁶ Additionally, there is a potential low affinity heme-binding site formed by a surface histidine residue, His₆₃, which may be capable of binding heme. Using Quick-Change mutagenesis, we not only demonstrated that the surface His₆₃ does not contribute to sensor heme binding in cells, but also identified 6 sensor variants that have heme affinities that may be ideal for sub-compartmental heme imaging through the systematic mutation of Met₇ and His₁₀₂ to alanine (Ala), cysteine (Cys), histidine (His), lysine (Lys), methionine (Met), serine (Ser), and tyrosine (Tyr). Taken together, our work has greatly expanded the available tool kit for heme imaging across cell types and subcellular compartments.

2.2 Experimental Methods

2.2.1 Yeast Strains, Genetic Manipulations, and Media

S. cerevisiae strains used in this study were derived from BY4741 (*leu2Δ0*). Yeast transformations were performed by the lithium acetate procedure.²⁷ Strains were maintained at 30°C on either enriched yeast extract-peptone based medium supplemented with 2% glucose (YPD), or synthetic complete medium (SC) supplemented with 2% glucose and leucine to maintain selection. Culturing of *hem1Δ* cells required supplementing SC media with 50 μg/mL of 5-aminolevulinic acid (5-ALA) or 15 mg/mL of ergosterol and 0.5% Tween-80.

2.2.2 Plasmid Construction and Mutagenesis

All plasmids were created from the cytosolic yeast HS1 sensor plasmid described previously by Hanna *et al.*¹ This is the p415-GPD plasmid, a low copy centromeric plasmid with the HS1 sensor driven by the GPD promoter.^{28,1} His₆₃, Met₇ and/or His₁₀₂ residues were mutagenized by Quick Change mutagenesis (Agilent Technologies). The numbering refers to its position in Cytochrome b562. His₆₃ was mutated to Asparagine (Asn) while Met₇ and His₁₀₂ were mutated to Ala, Cys, His, Lys, Met, Ser and Tyr. All plasmids and primers used are listed in the appendix as Table 2 and Table 3.

2.2.3 In-cell Fluorescence Spectroscopy

Cells were cultured in SCE-LEU media for ~14-16 hours to mid-exponential phase (OD₆₀₀ nm ~ 1-2.) After culturing, cells were harvested, washed in water, and resuspended in phosphate buffered saline (PBS) solution at a concentration of 3 OD₆₀₀/mL. Cellular sensor fluorescence emission was recorded on a Synergy Mx multi-modal plate reader using black Greiner Bio-one flat bottom fluorescence plates. EGFP and mKATE fluorescence were recorded using excitation and emission wavelength pairs of 488 nm and 510 nm and 588 nm and 620 nm, respectively, with 9 nm slit widths. For all measurements, cellular auto-fluorescence from both the EGFP and mKATE channels was subtracted by recording the fluorescence of cells not expressing the heme sensors.

2.2.4 In Situ Sensor Calibration

The fractional heme saturation of sensor variants was determined as described previously by Hanna *et al.*¹ Briefly, the fraction of heme bound to sensor is governed by the following relationship:

$$\text{Fractional Saturation} = \frac{R_{\text{expt}} - R_{\text{min}}}{R_{\text{max}} - R_{\text{min}}}$$

where R_{expt} is fluorescence ratio of the sensor in cells under any condition, R_{min} is the fluorescence ratio of the sensor when no heme is bound to it, and R_{max} is the fluorescence ratio when the sensor is 100% saturated with heme. R_{min} is derived from a parallel culture of *hemI* Δ cells, which lacks the 1st enzyme in the heme biosynthetic pathway, 5-ALA synthase, or from cells grown with 1 mM succinyl acetone, an inhibitor of the second enzyme in the heme biosynthetic pathway, 5-ALA dehydratase. Note, for all experiments described herein, R_{min} was derived from *hemI* Δ cells. R_{max} is derived from cells that are permeabilized with 200 ug/mL of digitonin and treated with 100 uM of hemin chloride and 1 mM ascorbate. An example of the determination of % bound for HS1 and HS1-M7A, two prototype sensors, is described in Figure 6. By considering R_{expt} , R_{min} , and R_{max} , the data indicate that HS1 and HS1-M7A are ~89% and ~48% bound with heme, respectively.

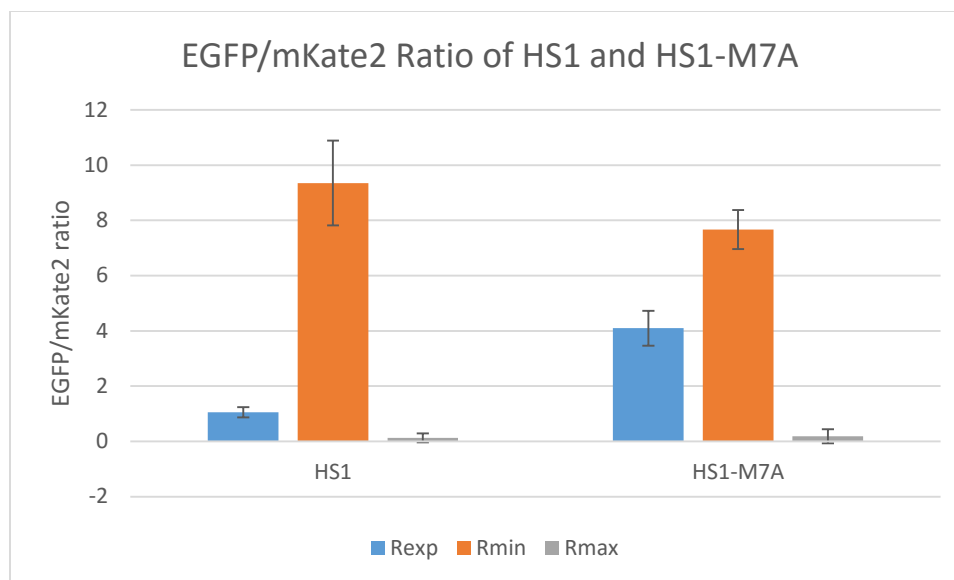


Figure 6. *In situ* calibration of heme sensors HS1 and HS1-M7A. EGFP/mKATE fluorescence ratios (R) of cells expressing HS1 or HS1-M7A. R_{exp} is derived from WT cells, R_{min} is derived from *hem1Δ* cells, and R_{max} is derived from WT cells that are permeabilized with digitonin and treated with excess heme and ascorbate as described in the text.

2.3 Results/Discussion

2.3.1 The effect of His₆₃ on Sensor Heme Binding

Cyt b₅₆₂ has a surface His residue, His₆₃, that may be a site for heme coordination.²⁶ In order to determine the impact of His₆₃ on sensor heme binding in cells, we mutated His₆₃ to Asn, and determined the fraction of heme bound to the sensor using the calibration approaches described in section 2.2. As shown in in Figure 7, the fractional saturation of HS1 and HS1-M7A is unaffected by the H63N. This demonstrates that the H63N mutation does not contribute to heme binding in cells.

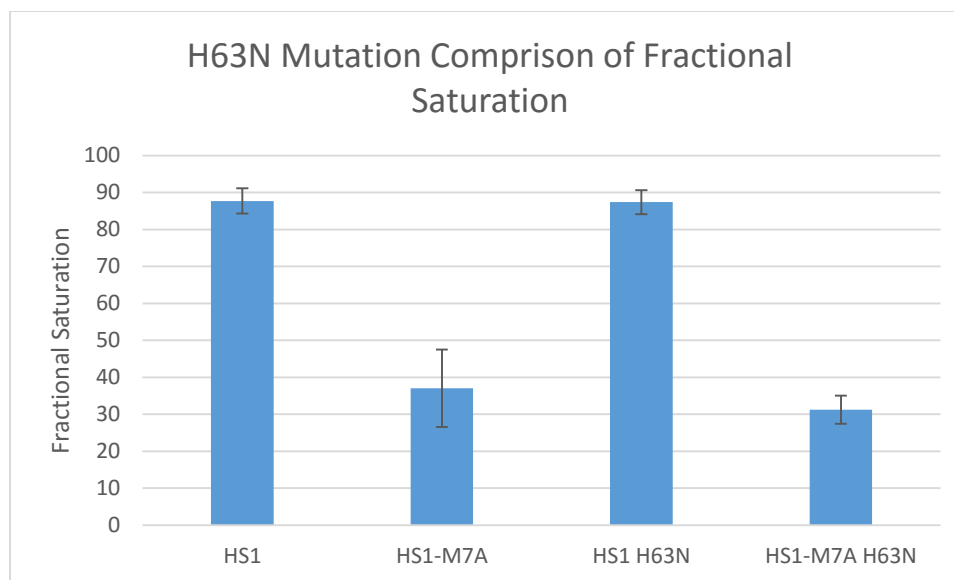


Figure 7. Fractional saturation of HS1, HS1-M7A, HS1 H63N and HS1-M7A H63N.

2.3.2 An Expanded Library of HS1 Heme Binding Site Mutants

In order to expand the range of heme binding affinities, we systematically mutated the Met₇ and His₁₀₂ heme-iron axial ligands to Ala, Cys, His, Lys, Met, Ser and Tyr, generating a library of ~47 new mutants. Upon introduction of these mutant sensors into yeast cells and fluorimetric determination of the fraction of heme bound to the sensors, we found that 20 variants had heme occupancies that resulted in 10-90 % heme bound (shown in green on Table 1) and 10 had heme occupancies below 10% or above 90% (shown in red on Table 1). 7 sensors have yet to be fully characterized and this work is ongoing (labeled untested in Table 1). Ten sensors were unable to be transformed into yeast for unknown reasons (labeled as not available (NA) in Table 1.)

For reference, the prototype sensors, HS1 and HS1-M7A, previously determined to be >90% or 20-50% bound with heme, are labeled yellow in Table 1. Sensor variants that have heme occupancies below 10% or above 90% are not particularly useful for

heme imaging in yeast because these sensors likely have heme binding affinities that are too low or too high for optimal heme imaging. However, these variants may be appropriate for other cell types of extra-cytosolic compartments.

Table 1. - Table of the fractional saturation of sensors from *in situ* calibration

Green = 10-90% saturated

Red = outside 10-90% saturated,

Yellow = HS1 and HS1-M7A

White = Untransformed (NA)/Untested

		Residue 7						
		Met	Lys	His	Cys	Tyr	Ser	Ala
Residue 102	Met	<10	30.6	NA	38.4	<10	NA	NA
	Lys	30.6	12.3	>90	<10	<10	NA	24.4
	His	93.0	63.8	29.3	>90	<10	NA	51.1
	Cys	60.9	53.3	81.6	Untested	19.6	NA	19.1
	Tyr	10.8	NA	37.2	Untested	<10	NA	Untested
	Ser	32.5	>90	Untested	Untested	<10	NA	62.1
	Ala	10.5	87.4	72.1	Untested	24.4	NA	Untested

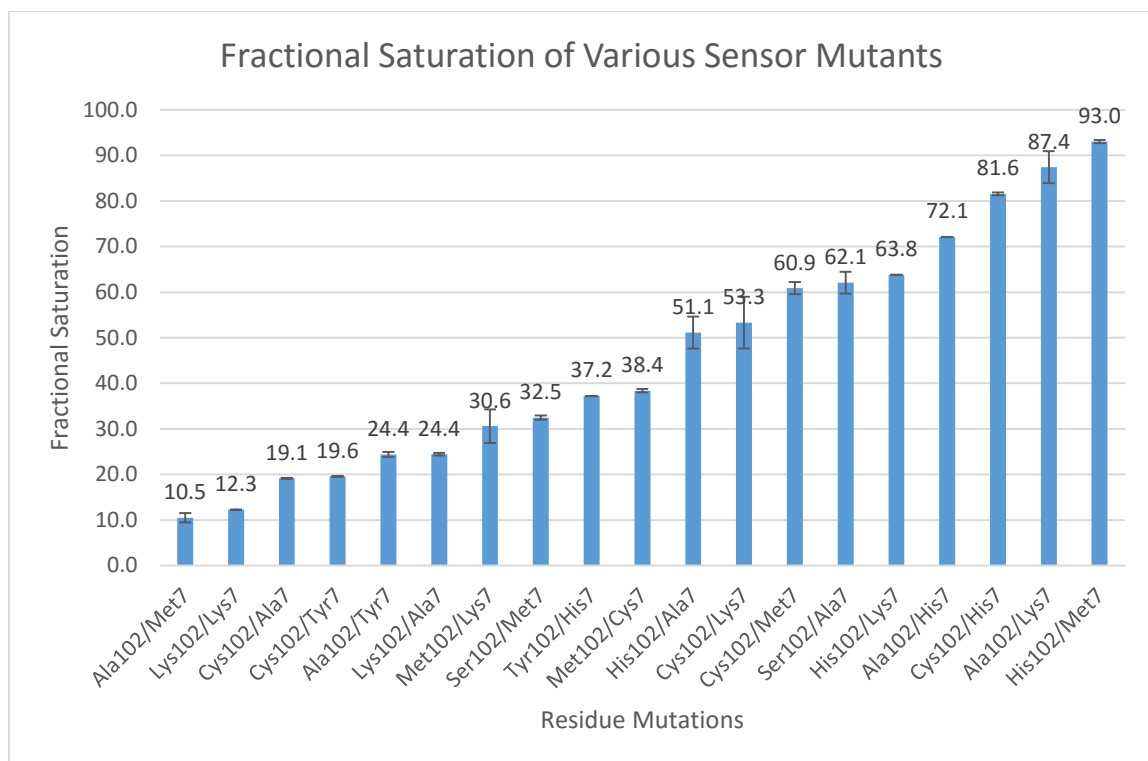


Figure 8. Fractional saturation of all sensor mutants in a range of 10-90% saturation, after *in situ* calibration of sensors.

It can be seen in both Table 1 and Figure 8 that the primary coordination site mutagenesis approach created a large range of sensors capable of binding cytosolic LH in yeast. This breadth of sensor affinities should allow for the detection of changes in LH in cellular compartments that were previously impossible to detect. Sensors with a fractional saturation that ranges between that of HS1 and HS1-M7A will be of use when looking at the LH pool in the nucleus and the mitochondria, something that HS1 and HS1-M7A were unable to do as they were completely saturated or unsaturated in these compartments, respectively. It is also likely that this range of sensor affinities will be useful when looking at heme dynamics in a range of cell types. Sensors with relatively

weak affinities, for example, will be of use in cell lines with a higher heme burden, e.g. blood and colon epithelial cells.²⁹

While most individual mutations do not appear to have a clear effect on sensor binding, there are several mutations that do, including mutating residue 7 to Ser and residue 7 to Tyr. It can be seen that the mutation of residue 7 to Ser possibly causes cytotoxicity, which is supported by the observation that all of these mutants were unable to be transformed. Mutating residue 7 to Tyr appears to significantly limit heme binding affinity as all sensors containing this mutation were below 25% saturated.

CHAPTER 3

CONCLUSION AND FUTURE WORK

3.1 Conclusion

Heme is a vital nutrient for cells despite its toxic nature; however, little is understood about how heme is regulated and trafficked in the cell. In recent years this has begun to be corrected with the development of heme sensors such as the one developed by the Reddi Lab. While these sensors are useful, they have significant limitations such as limited binding affinities, a limited color pallet, and limited preference for heme oxidation states. In an effort to broaden the binding affinities of these sensors and possibly create a sensor with preferential binding to oxidized heme, a series of mutations were made to the axial binding motif of HS1. Many of the mutations made appear to have binding affinities that range from virtually no binding to affinities on the order of HS1 itself. These results show that the development of a heme sensor library was a success in terms of creating a multitude of sensors with an expanded range of fractional heme occupancies. However, future work need to be done to determine sensor heme binding affinities and if these sensors can sense heme in compartments that HS1 and HS1-M7A were unable to sense heme in. Further, as heme can exist in primary oxidation states, ferric and ferrous, sensors with oxidation state specificity may uncover new aspects of the cell biology of labile heme pools. Altogether, the expanded library of heme sensors can be utilized to answer questions on how heme is regulated throughout the cell, and in particular, compartments like the mitochondria and the nucleus.

3.2 Future Work

Further *in vitro* characterization is required to guarantee that these sensors are working correctly and detecting heme as intended here. Answering questions of the actual heme binding affinities and preferences in heme oxidation state are important before these sensors can be used to answer questions *in vivo*. Once these sensors are confirmed to be working as they were intended it will be important to improve their design further. Altering the color pallet of theses sensor, for example, will be difficult but useful when considering looking at heme transportation throughout the cell. On the same vein, targeting these sensors to the organelles, such as the mitochondria and the nucleus, will likely be a fruitful endeavor as the concentration of labile heme in these compartments has been a difficult question to answer as of yet. Further development of these sensors to be used in various cell types, including bacterial and mammalian, will likely be a powerful step in understanding the questions of how heme effects human disease. Much is to be done to completely develop the tools necessary to answer all the questions of heme's role in biology, but the production of this library of sensors will be of tremendous use when tackling these questions.

APPENDIX A

PLASMIDS AND PRIMERS

Table 2. Description of plasmids used

Plasmid Name	Plasmid Description
pJA004-1	Expresses mKATEeGFPb562 (HS1 His102/Met7 H63N) Sensor
pJA005-1	Expresses mKATEeGFPb562 (HS1 His102/Ala7 H63N) Sensor
pJA006-1	Expresses mKATEeGFPb562 (HS1 Lys102/Ala7 H63N) Sensor
pJA007-1	Expresses mKATEeGFPb562 (HS1 Cys102/Ala7 H63N) Sensor
pJA008-1	Expresses mKATEeGFPb562 (HS1 Ser102/Ala7 H63N) Sensor
pJA009-1	Expresses mKATEeGFPb562 (HS1 Tyr102/Ala7 H63N) Sensor
pJA010-1	Expresses mKATEeGFPb562 (HS1 Ala102/Ala7 H63N) Sensor
pJA011-1	Expresses mKATEeGFPb562 (HS1 Met102/Ala7 H63N) Sensor
pJA012A-1	Expresses mKATEeGFPb562 (HS1 Lys102/Met7 H63N) Sensor
pJA013-1	Expresses mKATEeGFPb562 (HS1 Ser102/Met7 H63N) Sensor
pJA014-1	Expresses mKATEeGFPb562 (HS1 Cys102/Met7 H63N) Sensor
pJA015-1	Expresses mKATEeGFPb562 (HS1 Tyr102/Met7 H63N) Sensor
pJA016-1	Expresses mKATEeGFPb562 (HS1 Ala102/Met7 H63N) Sensor
pJA017A-3	Expresses mKATEeGFPb562 (HS1 Met102/Met7 H63N) Sensor
pJA018	Expresses mKATEeGFPb562 (HS1 His102/Lys7 H63N) Sensor
pJA019-1	Expresses mKATEeGFPb562 (HS1 His102/Tyr7 H63N) Sensor
pJA020	Expresses mKATEeGFPb562 (HS1 Met102/Lys7 H63N) Sensor
pJA021A-1	Expresses mKATEeGFPb562 (HS1 Ala102/His7 H63N) Sensor
pJA022-1	Expresses mKATEeGFPb562 (HS1 Met102/Ser7 H63N) Sensor
pJA023-1	Expresses mKATEeGFPb562 (HS1 Tyr102/His7 H63N) Sensor
pJA024-1	Expresses mKATEeGFPb562 (HS1 Lys102/Tyr7 H63N) Sensor
pJA025-1	Expresses mKATEeGFPb562 (HS1 Cys102/Tyr7 H63N) Sensor

pJA026A-1	Expresses mKATEeGFPb562 (HS1 Ser102/Tyr7 H63N) Sensor
pJA027A-1	Expresses mKATEeGFPb562 (HS1 Tyr102/Tyr7 H63N) Sensor
pJA028A-1	Expresses mKATEeGFPb562 (HS1 Ala102/Tyr7 H63N) Sensor
pJA029-1	Expresses mKATEeGFPb562 (HS1 Met102/Tyr7 H63N) Sensor
pJA030A-1	Expresses mKATEeGFPb562 (HS1 Met102/His7 H63N) Sensor
pJA031-1	Expresses mKATEeGFPb562 (HS1 Lys102/Lys7 H63N) Sensor
pJA032-1	Expresses mKATEeGFPb562 (HS1 Cys102/Lys7 H63N) Sensor
pJA033-1	Expresses mKATEeGFPb562 (HS1 Ser102/Lys7 H63N) Sensor
pJA034-1	Expresses mKATEeGFPb562 (HS1 Ala102/Lys7 H63N) Sensor
pJA035-1	Expresses mKATEeGFPb562 (HS1 Lys102/His7 H63N) Sensor
pJA036A-1	Expresses mKATEeGFPb562 (HS1 His102/His7 H63N) Sensor
pJA037A-1	Expresses mKATEeGFPb562 (HS1 Cys102/His7 H63N) Sensor
pJA038-1	Expresses mKATEeGFPb562 (HS1 Ser102/His7 H63N) Sensor
pJA039-1	Expresses mKATEeGFPb562 (HS1 His102/Cys7 H63N) Sensor
pJA040-1	Expresses mKATEeGFPb562 (HS1 Lys102/Cys7 H63N) Sensor
pJA041A-1	Expresses mKATEeGFPb562 (HS1 Met102/Cys7 H63N) Sensor
pJA042-1	Expresses mKATEeGFPb562 (HS1 Cys102/Cys7 H63N) Sensor
pJA043-1	Expresses mKATEeGFPb562 (HS1 Tyr102/Cys7 H63N) Sensor
pJA044A-1	Expresses mKATEeGFPb562 (HS1 Ser102/Cys7 H63N) Sensor
pJA045-1	Expresses mKATEeGFPb562 (HS1 Ala102/Cys7 H63N) Sensor
pJA046-1	Expresses mKATEeGFPb562 (HS1 Lys102/Ser7 H63N) Sensor
pJA047-1	Expresses mKATEeGFPb562 (HS1 His102/Ser7 H63N) Sensor
pJA048-1	Expresses mKATEeGFPb562 (HS1 Cys102/Ser7 H63N) Sensor
pJA049-1	Expresses mKATEeGFPb562 (HS1 Tyr102/Ser7 H63N) Sensor
pJA050-1	Expresses mKATEeGFPb562 (HS1 Ser102/Ser7 H63N) Sensor
pJA051-1	Expresses mKATEeGFPb562 (HS1 Ala102/Ser7 H63N) Sensor

Table 3. Primers used for QuikChange reactions

Primer	Sequence	Purpose
prRC008	GCAGATCTGGAAGACAACGCGGAAACTCTGAACG	mutates residue 7 -- > Ala in cytb562
prRC008-r	CGTTCAGAGTTTCCGCGTTGTCTTCCAGATCTGC	mutates residue 7 -- > Ala in cytb562
prRC009	CCACTCGTAATGCATATGCTCAGAAATACAGAGG	mutates residue 102 - -> Alain cytb562
prRC009-r	CCTCTGTATTTCTGAGCATATGCATTACGAGTGG	mutates residue 102 - -> Ala in cytb562
prRC010	CCACTCGTAATGCATATATGCAGAAATACAGAGG	mutates residue 102 - -> Met in cytb562
prRC010-r	CCTCTGTATTTCTGCATATATGCATTACGAGTGG	mutates residue 102 - -> Met in cytb562
pRH015	ACTCGTAATGCATATAAACAGAAATACAGAGGT	mutates residue 102 - -> Lys in cytb562
pRH015-r	TGGAGACATAAAGACAAATATACGTAATGCTCA	mutates residue 102 - -> Lys in cytb562
pRH016	GATCTGGAAGACAACAAGGAAACTCTGAACGAT	mutates residue 7 --> Lys in cytb562
pRH016-r	TAGCAAGTCTCAAAGGAACAACAGAAGGTCTAG	mutates residue 7 --> Lys in cytb562
pRH017	ACTCGTAATGCATATTGTCAGAAATACAGAGGT	mutates residue 102 - -> Cys in cytb562
pRH017-r	TGGAGACATAAAGACTGTTATACGTAATGCTCA	mutates residue 102-- > Cys in cytb562

pRH018	ACTCGTAATGCATATTCTCAGAAATACAGAGGT	mutates residue 102 - -> Ser in cytb562
pRH018-r	TGGAGACATAAAGACTCTTATACGTAATGCTCA	mutates residue 102-- > Ser in cytb562
pRH019	ACTCGTAATGCATATTATCAGAAATACAGAGGT	mutates residue 102 - -> Tyr in cytb562
pRH019-r	TGGAGACATAAAGACTATTATACGTAATGCTCA	mutates residue 102-- > Tyr in cytb562
pRH020	GATCTGGAAGACAACACTACGAACTCTGAACGAT	mutates residue 7 --> Tyr in cytb562
pRH020-r	TAGCAAGTCTCAAAGCATCAACAGAAGGTCTAG	mutates residue 7 --> Tyr in cytb562
prJA004	GCAGATCTGGAAGACAACCACGAACTCTGAACG	mutates residue 7 --> his in cytb562
prJA004-r	CGTTCAGAGTTTCGTGGTTGTCTTCCAGATCTGC	mutates residue 7 --> his in cytb562
prJA005	GCAGATCTGGAAGACAACCTCGGAACTCTGAACG	mutates residue 7 --> Ser in cytb562
prJA005-r	CGTTCAGAGTTTCCGAGTTGTCTTCCAGATCTGC	mutates residue 7 --> Ser in cytb562
prJA006	GCAGATCTGGAAGACAACCTGTGAACTCTGAACG	Mutates residue7 --> cys in cytb562
prJA006-r	CGTTCAGAGTTTCCAGTTGTCTTCCAGATCTGC	Mutates residue7 --> cys in cytb563

REFERENCES

1. Hanna, D. A.; Harvey, R. M.; Martinez-Guzman, O.; Yuan, X.; Chandrasekharan, B.; Raju, G.; Outten, F. W.; Hamza, I.; Reddi, A. R., Heme dynamics and trafficking factors revealed by genetically encoded fluorescent heme sensors. *Proceedings of the National Academy of Sciences* **2016**, 201523802.
2. Mayo, S. L.; Ellis, W. R.; Crutchley, R. J.; Gray, H. B., Long-range electron transfer in heme proteins. *Science* **1986**, 233 (4767), 948-952.
3. Lenfant, C.; Ways, P.; Aucutt, C.; Cruz, J., Effect of chronic hypoxic hypoxia on the O₂-Hb dissociation curve and respiratory gas transport in man. *Respiration physiology* **1969**, 7 (1), 7-29.
4. Dioum, E. M.; Rutter, J.; Tuckerman, J. R.; Gonzalez, G.; Gilles-Gonzalez, M.-A.; McKnight, S. L., NPAS2: a gas-responsive transcription factor. *Science* **2002**, 298 (5602), 2385-2387.
5. Gutteridge, J., Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clinical chemistry* **1995**, 41 (12), 1819-1828.
6. Chiabrando, D.; Vinchi, F.; Fiorito, V.; Mercurio, S.; Tolosano, E., Heme in pathophysiology: a matter of scavenging, metabolism and trafficking across cell membranes. *The Importance Of Iron In Pathophysiologic Conditions* **2015**, 132.
7. Hamza, I.; Dailey, H. A., One ring to rule them all: trafficking of heme and heme synthesis intermediates in the metazoans. *Biochimica Et Biophysica Acta (BBA)-Molecular Cell Research* **2012**, 1823 (9), 1617-1632.
8. Severance, S.; Hamza, I., Trafficking of heme and porphyrins in metazoa. *Chemical reviews* **2009**, 109 (10), 4596-4616.
9. Lara, F.; Lins, U.; Bechara, G.; Oliveira, P., Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick *Boophilus microplus*. *Journal of Experimental Biology* **2005**, 208 (16), 3093-3101.
10. Batista-Nascimento, L.; Pimentel, C.; Andrade Menezes, R.; Rodrigues-Pousada, C., Iron and neurodegeneration: from cellular homeostasis to disease. *Oxidative medicine and cellular longevity* **2012**, 2012.
11. Sesink, A. L.; Termont, D. S.; Kleibeuker, J. H.; Van der Meer, R., Red meat and colon cancer the cytotoxic and hyperproliferative effects of dietary heme. *Cancer research* **1999**, 59 (22), 5704-5709.

12. Duffy, S. P.; Shing, J.; Saraon, P.; Berger, L. C.; Eiden, M. V.; Wilde, A.; Tailor, C. S., The Fowler syndrome-associated protein FLVCR2 is an importer of heme. *Molecular and cellular biology* **2010**, *30* (22), 5318-5324.
13. Hebert, L. E.; Weuve, J.; Scherr, P. A.; Evans, D. A., Alzheimer disease in the United States (2010–2050) estimated using the 2010 census. *Neurology* **2013**, *80* (19), 1778-1783.
14. Ghosh, C.; Seal, M.; Mukherjee, S.; Ghosh Dey, S., Alzheimer's Disease: A Heme–A β Perspective. *Accounts of Chemical Research* **2015**, *48* (9), 2556-2564.
15. Schipper, H.; Cisse, S.; Stopa, E., Expression of heme oxygenase-1 in the senescent and alzheimer-diseased brain. *Annals of neurology* **1995**, *37* (6), 758-768.
16. Pramanik, D.; Ghosh, C.; Dey, S. G., Heme–Cu bound A β peptides: spectroscopic characterization, reactivity, and relevance to Alzheimer's Disease. *Journal of the American Chemical Society* **2011**, *133* (39), 15545-15552.
17. Atamna, H., Heme binding to Amyloid- β peptide: Mechanistic role in Alzheimer's disease. *Journal of Alzheimer's Disease* **2006**, *10* (2, 3), 255-266.
18. Heavey, P. M.; McKenna, D.; Rowland, I. R., Colorectal cancer and the relationship between genes and the environment. *Nutrition and cancer* **2004**, *48* (2), 124-141.
19. IJssennagger, N.; Derrien, M.; van Doorn, G. M.; Rijnierse, A.; van den Bogert, B.; Müller, M.; Dekker, J.; Kleerebezem, M.; van der Meer, R., Dietary heme alters microbiota and mucosa of mouse colon without functional changes in host-microbe cross-talk. *PLoS One* **2012**, *7* (12), e49868.
20. Dean, K. M.; Qin, Y.; Palmer, A. E., Visualizing metal ions in cells: an overview of analytical techniques, approaches, and probes. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* **2012**, *1823* (9), 1406-1415.
21. Song, Y.; Yang, M.; Wegner, S. V.; Zhao, J.; Zhu, R.; Wu, Y.; He, C.; Chen, P. R., A Genetically Encoded FRET Sensor for Intracellular Heme. *ACS chemical biology* **2015**, *10* (7), 1610-1615.
22. Arpino, J. A.; Czapinska, H.; Piasecka, A.; Edwards, W. R.; Barker, P.; Gajda, M. J.; Bochtler, M.; Jones, D. D., Structural basis for efficient chromophore communication and energy transfer in a constructed didomain protein scaffold. *Journal of the American Chemical Society* **2012**, *134* (33), 13632-13640.
23. Pletnev, S.; Shcherbo, D.; Chudakov, D. M.; Pletneva, N.; Merzlyak, E. M.; Wlodawer, A.; Dauter, Z.; Pletnev, V., A crystallographic study of bright far-red

fluorescent protein mKate reveals pH-induced cis-trans isomerization of the chromophore. *Journal of Biological Chemistry* **2008**, 283 (43), 28980-28987.

24. Sassa, S., Why heme needs to be degraded to iron, biliverdin IX α , and carbon monoxide? *Antioxidants & redox signaling* **2004**, 6 (5), 819-824.

25. Chakravarti, R.; Aulak, K. S.; Fox, P. L.; Stuehr, D. J., GAPDH regulates cellular heme insertion into inducible nitric oxide synthase. *Proceedings of the National Academy of Sciences* **2010**, 107 (42), 18004-18009.

26. Hay, S.; Wydrzynski, T., Conversion of the Escherichia coli Cytochrome b 562 to an archetype cytochrome b: A mutant with bis-histidine ligation of heme iron. *Biochemistry* **2005**, 44 (1), 431-439.

27. Gietz, R. D.; Schiestl, R. H., Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. *Yeast* **1991**, 7 (3), 253-263.

28. Mumberg, D.; Müller, R.; Funk, M., Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **1995**, 156 (1), 119-122.

29. Khan, A. A.; Quigley, J. G., Control of intracellular heme levels: heme transporters and heme oxygenases. *Biochimica Et Biophysica Acta (BBA)-Molecular Cell Research* **2011**, 1813 (5), 668-682.